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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No,	920905.90041
First Inventor or Application Identifier	Katherine W. Osteryoung
Title	Manipulation of Min Genes in Plants
Express Mail Label No.	EK290771354US

JC811 U.S. PTO 09/551243 04/19/00

APPLICATION ELEMENTS

See MPEP Chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231**

1 [X] Fee transmittal Form (Submit an original and a duplicate for fee processing)

2 [X] Specification [Total 25]
(preferred arrangement set forth below)
- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed Sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings *(if filed)*
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3 [X] Drawing(s) (35 USC 113) [Total Sheets 3]

4. Oath or Declaration [Total Pages 2]

a. [X] Newly unexecuted (original or copy)

b. [ ] Copy from prior Application (37 CFR 1.63(d)) (for continuation/divisional with Box 17 completed)

[Note Box 5 below]

i. [ ] DELETION OF INVENTOR(S) Signed Statement attached deleting inventor(s) named in prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

5 [ ] Incorporation By Reference (useable if Box 4b is checked) The entire disclosure of the prior application from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference herein.

6. [ ] Microfiche Computer Program *(Appendix)*

7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*

[X] Computer readable Copy
[X] Paper Copy (identical to computer copy)
[X] Statement Verifying identity of above

ACCOMPANYING APPLICATION PARTS

8 [ ] Assignment Papers (cover sheet & documents)

9 [ ] 37 CFR 3.73(b) Statement [ ] Power of Attorney (where there is an assignee)

10 [ ] English Translation Document *(if applicable)*

11 [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations

12 [ ] Preliminary Amendment

13 [X] Return receipt postcard (MPEP 503) *(Should be specifically itemized)*

14 [ ] *Small Entity Statement(s) [ ] Statement filed in prior application Status still proper and desired

15 [ ] Certified copy of priority Document(s) *(if foreign priority is claimed)*

16 [ ] Other:

* A new statement is required to pay small entity fees, except where one has been filed in a prior application and is being relied upon

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application no. 60/130,403

Prior application information: Examiner: Group/Art Unit:

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April 19, 2000

Assistant Commissioner of Patents
Box Patent Application
Washington DC 20231

Re: Filing New Patent Application

Dear Sir:

Enclosed for filing please find a new patent application entitled:

MANIPULATION OF MIN GENES IN PLANTS

by Katherine W. Osteryoung

The undersigned hereby certifies that this document is being deposited with the United States Postal Service today, April 19, 2000, by the "Express Mail" service, utilizing Express Mail label number EK290771354US, addressed to: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

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Respectfully submitted,

Enclosures
QBMAD\216467

MANIPULATION OF MIN GENES IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from provisional patent application Serial No. 60/130,403 filed April 19, 1999.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

The modern agricultural industry has devoted considerable resources toward the development of phenotypically distinct plants with economically advantageous qualities. Valuable features in food crops include increased vigor, disease resistance, greater yields, extended shelf-life, and enhanced nutritional content.

The development of high yielding food crops is particularly important. Each year, the tillable land available for agricultural production is reduced as more acreage is devoted to alternative uses. At the same time, the human population is rapidly increasing. Therefore, it is essential to increase agricultural productivity in order to meet the nutritional needs of the world's burgeoning population.

Efforts to develop crop plants that produce higher yields have been directed toward pest control, or toward the selection and breeding of varieties that bear greater numbers of fruit, or that produce larger fruit. These crop breeding endeavors are very time-consuming and labor-intensive, but have historically increased crop yields incrementally over time. Modern techniques of recombinant DNA manipulation and genetic engineering offer the prospect of the more rapid creation of new plant varieties with novel traits. The creation of genetically modified, or transgenic, plants with altered phenotypes arising from artificially inserted genetic constructions has become a

common practice in modern agriculture.

If one is going to genetically engineer plants, the genetic engineering or recombinant DNA manipulation of plastids is one area in which improvements to plants might be targeted. Plastids are membrane-delimited organelles in plant cells which are essential for sustaining plant growth and cell viability. They are the site for the synthesis of essential amino acids, vitamin E, pro-vitamin A, starch, certain growth hormones, lipids, and pigments such as carotenes, xanthophylls, and chlorophylls. In plants, plastids include chloroplasts, chromoplasts, leucoplasts and amyloplasts, which are typically found in all organs of the plant including its leaves, roots, stems, petals, and seeds.

The specialized plastid chloroplast is where photosynthesis occurs. Photosynthesis in plants is an important biosynthetic process upon which virtually all living organisms depend for our very existence. During photosynthesis, energy in the form of light is converted to ATP, which fuels a series of enzymatic reactions that catalyze the synthesis of carbohydrates, which are further used for metabolic energy in the plant. Photosynthesis also produces molecular oxygen (O_2) is a byproduct. Because photosynthesis is the source of metabolic energy in plants, photosynthetic efficiency is a significant factor associated with general plant growth and vigor. Chloroplasts also synthesize amino acids and lipids.

U.S. Patent 5,981,836, incorporated herein by reference, discloses genetic constructs capable of altering the number and size of plastids in plant cells. These construct contain an *Arabidopsis* plastid division FtsZ protein coding sequence and a promoter, not natively found associated with the FtsZ protein coding sequence, which promotes expression of the *Arabidopsis* plastid division FtsZ protein coding sequence in the plant. The FtsZ protein is a bacterial cytoskeletal protein and structural homologue of tubulin that polymerizes on the inner surface of the cytoplasmic membrane to forms a cytokinetic ring during cell division. Transgenic expression of the coding sequence results in a high percentage of novel phenotypes characterized by alterations in the number and size of plastids in the cells of the plant in which the construct is expressed.

There is also on-going efforts to make transgenic plants that are more suited for particular applications or which have transgenes inserted into them to have localized

effects inside the cells of the plants. For example, there are a number of transgenes inserted into plants which maximize the usefulness of the inserted traits if the transgenes are transformed into the chloroplasts of the plant. Since one method for chloroplast transformation is based on the delivery of transgenes coated onto small carrier particles into the interior of the chloroplasts themselves, this technique is easier to perform if the chloroplasts themselves are larger than normal. So one technique that would be useful for this effort is to make plants with larger chloroplasts.

What is needed in the art are additional means for altering the shape, size and/or number of chloroplasts and other plastids in agronomically and horticulturally important plants to achieve greater plant productivity and nutritional quality.

BRIEF SUMMARY OF THE INVENTION

The present invention is a plant comprising in its genome a genetic construction including a sense or antisense MinD protein coding sequence and a promoter, not natively associated with the MinD protein coding sequence, which promotes expression of the sequences in the plant, wherein expression of the sequence in the plant causes alterations in the number, shape and/or size of the plastids in the plant cells of the plant. The present invention also discloses a method for altering the number, shape and/or size of the plastids using the genetic construct described above.

The present invention also includes DNA sequences (SEQ ID NO:1 and SEQ ID NO:3) representing genes that function in regulating plastid division, and which, when ectopically expressed, alters the number, shape and/or size of chloroplasts and other types of plastids present in plant cells.

The present invention is also directed toward a genetic construct including a MinD protein coding sequence and a promoter that promotes expression of the sequence in plants, the promoter not being natively associated with the MinD protein coding sequence.

The present invention is also a seed, including in its genome a genetic construct comprising a MinD protein coding sequence and a promoter, not natively associated with the MinD protein coding sequence, that promotes gene expression in plants.

The present invention is also a plant cell including in its genome a genetic

construct comprising a MinD protein coding sequence and a promoter, not natively associated with the MinD protein coding sequence, that promotes gene expression in plants.

It is an object of the present invention to provide a transgenic plant that has a novel phenotype with advantageous qualities, including decreased numbers of enlarged chloroplasts.

Other objects, advantages, and features of the present invention will become apparent after review of the specification and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 is an illustration of the alignment of MinD proteins from several photosynthetic organisms.

Fig. 2 is a model showing the proposed effect of plastid-localized *AtMinD1* on the positioning of the plastid division apparatus.

Fig. 3 are graphs illustrating the frequency distribution of chloroplast sizes in mesophyll cells from *Arabidopsis* wild-type plants and *Arabidopsis* plants transformed by an antisense *AtMinD1* construct.

DETAILED DESCRIPTION OF THE INVENTION

It is disclosed here that nuclear-encoded, plastid-targeted forms of genes encoding a MinD protein have been identified in plants, and these genes have been shown to play an important role in the division of plastids. The MinD genes had previously only been demonstrated to exist in prokaryotes. The data presented here demonstrates that all plants natively have MinD genes, and that the function of the endogenous genes can be altered by genetic engineering.. Reduced expression of an endogenous MinD gene in a transgenic plant results in asymmetric plastid division leading to an abnormally heterogeneous distribution of plastid numbers and sizes in plant cells. Overexpression of the MinD gene in a transgenic plant results in the inhibition of plastid division and fewer numbers of large plastids.

One aspect of the present invention is a plant that contains in its genome a genetic construct having a sense or antisense plant MinD protein coding sequence and a

promoter, not natively associated with the MinD protein coding sequence, which promotes expression of the sequence in plant cells. Insertion of the genetic construction results in plants having a high percentage of novel phenotypes characterized by alterations in the number, shape and/or size of plastids in cells of the plant in which the construct is expressed.

The identification and characterization of two initial MinD coding sequences from plants that are useful in the present invention are described in the examples below. The sequence designated AtMinD (At for *Arabidopsis thaliana*, SEQ ID NO:1) was identified by BLAST similarity searching on the basis of homology to bacterial MinD genes. The sequence designated TeMinD (Te for *Tagetes erecta*, SEQ ID NO:3) was identified by cDNA library screening on the basis of homology to bacterial MinD genes.

It should be understood that the initial plant MinD genes, the identification of which are described here, were identified based on sequence comparison to the analogous genes known in bacteria. Since the bacterial gene sequence was sufficient to permit the identification of plant MinD genes, and since the plant MinD will be more closely related to each other than they are to bacterial genes, the data presented here make possible the recovery of the respective MinD gene from most, if not all, plant species.

Based on analogous function to the bacterial proteins, plant MinD proteins are involved in the placement of the FtsZ ring during plastid division. The plant MinD genes are believed to descend from a key prokaryotic cell division mechanism through the evolution of photosynthetic eukaryotes. The process of cell division in bacteria is mediated by a set of at least ten proteins that assemble into a macromolecular complex at the cell midpoint. Chief among these is the bacterial cell division protein FtsZ, a prokaryotic cytoskeletal protein and structural homologue of tubulin that polymerizes on the inner surface of the cytoplasmic membrane to forms a contractile ring. Assembly of the FtsZ ring is the earliest known step in formation of the bacterial division complex.

The mechanism by which placement of the FtsZ ring is determined in bacteria is still uncertain, but genetic studies have uncovered some of the critical players. In *E. coli*, precise localization of the FtsZ ring at the cell center is established by the Min system of proteins, comprising MinC, MinD, and MinE. In mutants lacking MinC or

MinD, the FtsZ ring is frequently misplaced near one of the cell poles such that cell division results in the formation of nonviable "minicells" which lack chromosomes and cannot expand. Thus, MinC and MinD act in wild type cells by inhibiting FtsZ ring formation at polar sites and restricting the ring to the midcell. This activity in *E. coli* involves an oscillation of both MinC and MinD from one cell pole to the other. MinE, which is targeted independently of FtsZ to a medial ring, prevents MinC and MinD from localizing at the midcell, thereby allowing the FtsZ ring to assemble specifically at this position.

In *Bacillus subtilis*, MinC and MinD also prevent FtsZ ring assembly at polar sites, but are localized at both poles simultaneously and do not oscillate. *B. subtilis* lacks MinE, relying instead on a different protein, DivVIA, to tether MinC and MinD to the cell poles. Though the mechanisms restricting the activity of MinC and MinD to polar sites are different in *E. coli* and *B. subtilis*, in both cases the absence of these proteins at the midcell establishes the site of FtsZ ring assembly, and MinD is required for the proper localization and division-inhibiting activity of MinC.

In plants and other photosynthetic eukaryotes, constriction of the chloroplast during division usually occurs at the middle of the plastid perpendicular to the long axis. See the diagram of Figure 2. These observations indicate that the positioning of the plastid division machinery in plants, like the positioning of the FtsZ ring in bacteria, is a carefully regulated process. The discovery of the existence of a nuclear gene from *Arabidopsis* and *Tagetes* encoding a chloroplast-targeted homologue of MinD and the examination of the relationship between chloroplast shape, size and number in transgenic plants indicates that a Min-based system operates in specifying placement of the plastid division components in plant cells during plastid division.

As used herein, "MinD" refers to the *Arabidopsis* MinD protein coding sequence (SEQ ID NO: 2) and the *Tagetes erecta* (Marigold) MinD protein coding sequence (SEQ ID NO: 4), as well as the analogous gene sequences from other plants as well as variations and mutants thereof which retain plastid division control functionality. As shown in Figure 1, the MinD proteins are highly conserved among diverse species capable of conducting photosynthesis. It is expected that all plants contain MinD genes homologous to the *Arabidopsis* and *Tagetes* genes. The bacterial MinD protein is also

homologous to the plant MinD genes and can be used as well in transgenic plants. Given the apparent ubiquitousness and high degree of conservation of the MinD proteins among plant species, it is reasonable to expect that MinD genes, of which the AtMinD and the TeMinD genes are but two examples, from any plant could be used in the practice of the present invention. For example, MinD genes from plants that are raised for their agricultural or horticultural value may be used in the practice of the present invention. It can be expected, from the sequence data presented below, that any plant MinD have at least 50%, and more likely at least 80%, sequence identity at the amino acid level with either the *Arabidopsis* or the *Tagetes* MinD protein sequence. The *Arabidopsis* and *Tagetes* MinD sequences compared to each other have a sequence identity at the amino acid level of 92%, a high degree of sequence identity. By sequence identity it is meant that at least the defined percentage of amino acid residues in the proteins being compared have the same identity and are located in the same sequence order as the corresponding amino acids in the protein to which it is being compared. A useful calculation (as used here) for amino acid sequence identity comparison is done using pairwise comparisons using the SIM local alignment algorithm (Huang and Miller (1991)) with the default parameters specified on the ExPASy Molecular Biology Server, Swiss Institute of BioInformatics. The comparison at the amino acid level means that two genes being compared may have nucleotide sequences that differ more greatly than the amino acid sequence, which is possible given the degeneracy of the genetic code, but that they encode amino acids which have the requisite degree of sequence identity.

It is specifically contemplated that any MinD protein coding sequence could be used in the practice of the present invention. "MinD protein coding sequence" is defined to include any plant DNA sequence capable of overexpressing or reducing the activity of the MinD gene native to the plant in which the MinD protein coding sequence is introduced. A MinD protein coding sequence may be an unmodified genomic entire gene sequence isolated from any plant, a cDNA sequence derived from any plant, a genomic or cDNA sequence that is modified to contain minor nucleotide additions, deletions, or substitutions, or a synthetic DNA sequence. The term is intended to apply, as well, to analogous sequences from other plants as well as allelic variations and mutations which are still capable of controlling plastid division.

By "plastid division activity" it is meant the ability to cause alterations in the number or size of the chloroplast or other types of plastids present in cells of a transgenic plant in which the MinD protein coding sequence is expressed.

By "transgene" it is meant to describe an artificial genetic construction carried in the genome of a plant and inserted in the plant or its ancestor by gene transfer. Such transgenes are transmissible by normal Mendelian inheritance once inserted into the genome of a parental plant.

It is specifically envisioned that transgenic plants can be made with a transgene for a MinD protein coding sequence which selectively either up-regulates or down-regulates plastid MinD division activity. For fewer plastid divisions, extra copies or high expression copies of MinD protein coding sequence transgenes are inserted into plants, resulting in fewer and larger plastis in the transgenic plants. Sometimes high expressing plants will produce only one or a very fewer chloroplasts per cell. For more plastid division activity, the use of an antisense MinD protein coding sequence transgene, or any other gene inhibition technique, may be used to down regulate plastid division activity resulting in a greater variability in plastid number and size. Both up and down regulation of plastids will be useful for certain applications.

Transgenic *Arabidopsis* plants were obtained as a model system using the *Agrobacterium* transformation system, as described in the examples. *Arabidopsis* is often used as a model plants in such experiments because of the relatively small size of its genome and also because it is a small compact plant easy to grow and easy to conduct experiments on. *Agrobacterium*-mediated transformation is used since it is known to work well with many dicot plants and some monocots. Other methods of transformation equally useful in dicots and monocots may also be used in the practice of the present invention. Transgenic plants may be obtained by particle bombardment, electroporation, or by any other method of transforming plants known to one skilled in the art of plant molecular biology. The experience to date in the technology of plant genetic engineering is that the method of gene introduction is not of particular importance in the phenotype achieved in the transgenic plant.

The present invention is also directed toward a genetic construct comprising a MinD protein coding sequence and a promoter, not natively associated with the

sequence, which promotes expression of the MinD protein coding sequence in plants at levels sufficient to cause novel phenotypes. The construct may contain the sequence in either the sense or antisense orientation. The development of constructs that have been found to alter the number or size of chloroplasts in transformed plant cells is described in the examples below. Briefly, relevant features of these constructs include a kanamycin resistance marker and, in 5' to 3' order, the CamV 35S promoter operably connected to a chloroplast division sequence, and a transcriptional terminator, or polyadenylation sequence, from an Agrobacterium gene known as OCS.

The CaMV 35S promoter is a constitutive promoter known to function in a wide variety of plants. Other promoters that are functional in the plant into which the construct will be introduced may be used to create genetic constructs to be used in the practice of the present invention. These may include other constitutive promoters, tissue-specific promoters, developmental stage-specific promoters, and inducible promoters. Promoters may also contain certain enhancer sequence elements that improve the efficiency of transcription.

The examples below describe the use of an expression vector that contains a kanamycin resistance gene as a selectable marker for selection of plants that have been transformed with the genetic construct. Numerous selectable markers, including antibiotic and herbicide resistance genes, are known in the art of plant molecular biology and may be used to construct expression vectors suitable for the practice of the present invention. Expression vectors may be engineered to include screenable markers, such as beta glucuronidase (GUS).

The genetic constructs employed in the examples below were engineered using the plasmid vector pART27 (Gleave, Plant Mol. Biol. 20:1203-1207, 1992). It is anticipated that other plasmid vectors or viral vectors, or other vectors that are known in the art of molecular biology, will be useful in the development of a construct that may be used to transform a plant to obtain expression of a MinD protein coding sequence. The creation of a genetic construct suitable for transformation using the *Agrobacterium* system is described, however, any transformation system for obtaining transgenic plants may be used. The construction of a vector and the adaptation of that vector to a particular transformation system are both within the ability of one skilled in the art.

The present invention also contemplates a method for altering the shape, size and/or number of plastids in a plant, relative to the wild type plant. The method comprises the steps of making a genetic construct comprising a MinD protein coding sequence and a promoter, not be natively associated with the sequence, transforming the plant with the genetic construct, and growing the transgenic plant so created as to allow expression of the genetic construct. The genetic construct as a transgene in the plant will change the size, shape and/or number of the plastids in the plant cells of the plant.

Alterations in plastid size, shape and/or number via genetic engineering of MinD expression in accordance with the present invention has the potential to result in improved productivity or increased vigor due to enhanced photosynthetic capacity and allow enhanced production of commercially important compounds that accumulate naturally or as a result of genetic engineering. The ability to alter the expression of the chloroplast division genes allows the manipulation of the size and number of chloroplasts in plant cells. Because chloroplast number is known to have a direct effect on photosynthetic capacity, it is likely that by manipulating levels of plastid division proteins in genetically engineered plants to achieve increased numbers or size of plastids, one may obtain plants having advantageous properties.

In the examples below, changes in chloroplast numbers and size were examined in plants in which a MinD protein coding sequence was expressed as a transgene in transgenic plants. It is expected that MinD protein coding sequences are also involved in regulating the division of other plastids, including chromoplasts, amyloplasts, and leucoplasts. These plastids are of great agronomic importance because they synthesize carotenoids, starch, and oils. Manipulation of the expression of chloroplast division sequences to alter the number or size of plastids other than chloroplasts is within the scope and spirit of the present invention.

Figure 2 sets forth a model for the structural organization of the plastid division apparatus in plants in which plastid division is mediated by two FtsZ-containing plastid-dividing rings, one localized on the stromal surface of the inner chloroplast envelope membrane containing FtsZ1, and the other on the cytosolic surface of the outer envelope membrane containing FtsZ2 (Figure 2, Panel A). Implied in this model is the coordinated positioning of division components across the envelope at the plastid

midpoint. In wild-type plants, both stromal and cytosolic PD rings, proposed to contain FtsZ1 and FtsZ2, respectively, are localized at the plastid midpoint, and the coordinated constriction of the two rings results in symmetric division yielding two daughter plastids approximately equal in size (Figure 2, panel A). Antisense repression of MinD leads to misplacement of the stromal FtsZ1 ring in many, though not necessarily all, plastids. When it does occur, the cytosolic FtsZ2 ring in turn becomes localized to a site on the outer membrane corresponding to the site of misplacement of the FtsZ1 ring (Figure 2, panel B, right). Other components of the two PD rings presumably also assemble at this position. The coordinated action of the two mislocalized PD rings results in a productive but asymmetric division event, yielding daughter plastids of unequal size. Multiple rounds of plastid division in which the stromal FtsZ1 ring was sometimes but not always misplaced could further increase the size variability.

The nonlimiting examples that follow are intended to be purely illustrative.

EXAMPLES

Isolation and Characterization of a MinD gene in *Arabidopsis thaliana*.

An homolog of the bacterial MinD gene was isolated from *Arabidopsis thaliana* as follows. The amino acid sequence of *Chlorella vulgaris* MinD was used as a query sequence to search the nonredundant GenBank database using the TBLASTN algorithm. A highly significant match was found to an open reading frame in the P1 library clone MZF18 (accession number AB009056) from chromosome V of *Arabidopsis*. This Arabidopsis DNA sequence, designated *AtMinD1*, is shown in SEQ ID NO:1. It contains an open reading frame (ORF) spanning nucleotides 32,980 through 33,957 on the minus strand of MZF18, which is uninterrupted by introns and encodes a polypeptide of 326 amino acids with a calculated molecular weight of 35,690. This Arabidopsis MinD amino acid sequence, designated AtMinD, is shown in SEQ ID NO:2.

Fig. 1 illustrates a sequence comparison by sequence alignment between the sequence from *Arabidopsis* and the corresponding gene sequences from other photosynthetic organisms. The sequences used in the alignment are identified as follows: *Sy, Synechocystis* PCC6803, Q55900; *Gt, Guillardia theta* (plastid genome),

AAC35621; *Cv, Chlorella vulgaris* (plastid genome), P56346; *Pw, Prototheca wickerhamii* (plastid genome), CAB53105; *No, Nephroselmis olivacea* (plastid genome), AAD54908; *At, Arabidopsis thaliana*, AB009056 (translated sequence of nucleotides 32,980 through 33,957, minus strand); *Os, Oryza sativa* AF149810 (partial sequence). Only the first 163 amino acids of the *O. sativa* sequence were used in the alignment. Dashes (-) indicate gaps in the alignment. Gaps at the amino termini were removed manually. Identical amino acids among the sequences shown are boxed in black. Asterisks (*) indicate residues identical among all proteins when the following bacterial MinD sequences are added to the alignment (not shown): *Bacillus subtilis*, Q01464; *Escherichia coli*, BAA36022; *Helicobacter pylori* 26695, AAD07400; *Deinococcus radiodurans*, AAF10331; *Aquifex aeolicus*, AAC06996.

This alignment of several MinD amino acid sequences in Fig. 1 from various photosynthetic organisms was performed using CLUSTAL W 1.8. The alignment revealed regions of high sequence similarity, indicating that the gene has been highly conserved during the evolution of chloroplasts. The MinD protein encoded by *AtMinD1* shares a 65% identity with the MinD protein from *Chlorella vulgaris* (P56346), a slightly lower extent of identity (58-62%) with the MinD proteins encoded in the plastid genomes of *Guillardia theta* (AAC35621), *Prototheca wickerhamii* (CAB53105), *Nephroselmis olivacea* (AAD54908) and *Oryza sativa* (AF149810), and a 53% identity with the MinD protein from the photosynthetic prokaryote *Synechocystis* (PCC6803, Q55900). AtMinD also shares a greater than 40% amino acid identity with the bacterial MinD sequences of *Bacillus subtilis* (Q01464); *Escherichia coli* (BAA36022); *Helicobacter pylori* 26695 (AAD07400); *Deinococcus radiodurans* (AAF10331); and *Aquifex aeolicus* (AAC06996), data not shown. Sequence identity was calculated using the SIM local alignment algorithm (Huang and Miller 1991) with the default parameters specified on the ExPASy Molecular Biology Server, Swiss Institute of BioInformatics (http://expasy.hcuge/sprot/sim-prot.html).

Isolation and Characterization of a MinD gene in *Tagetes erecta* (Marigold).

An homolog of the bacterial MinD gene was isolated from *Tagetes erecta* (Marigold) as follows. Color complementation was used to screen a cDNA library made

from poly A RNA isolated from stage 3 and 4 petals (approximately 8-10 mm and 13-15 mm in length, respectively) taken from the marigold variety Dark Orange Lady (W. Atlee Burpee Company, Clinton, Iowa). This technique relies on the ability of an *E. coli* engineered to express certain carotenoids to accumulate the carotenoids when carotenoid biosynthetic genes are expressed from a plasmid. A second plasmid from a library of interest can be introduced into this background with a different selectable marker and a compatible replicon which enables the selection of colonies having a desired phenotype. This color complementation method has proven to be effective for isolating carotenoid biosynthetic genes from a number of organisms.

To construct the cDNA library, total RNA from the marigold petals was isolated by LiCl precipitation and poly A RNA was obtained by two passes of the RNA over an oligo dT cellulose column. (Stratagene, La Jolla, CA). Single clone excision and mass excision of the lambda library to yield phagemids were then performed as recommended by the manufacturer. Isolated plasmids were sequenced by primer walking on both strands using a dRhodamin cycle sequencing kit.

E. coli cells (strain DH5α) containing the plasmid pAC-ZEAX for producing zeaxanthin were transformed with plasmids containing cDNA from the Marigold library, and then grown in LB supplemented with chloramphenicaol to maintain the carotenoid-gene containing plasmid and with ampicillin (100 μg/ml) to maintain the marigold library plasmids.

A low temperature screen led to the isolation of a marigold gene encoding the plastid division protein MinD. This screen was based on the observation that *E. coli* genetically engineered to accumulate zeaxanthin grew more slowly at 18°C than *E. coli* harboring the vector plasmid alone and accumulated significantly less zeaxanthin than when grown at 37°C. The basis of this temperature dependent phenotype is unknown. When the marigold cDNA library was transformed into zeaxanthin containing *E. coli*, numerous rapidly growing, highly pigmented colonies were identified in a background of pale, slow growing colonies. Plasmids isolated from several of these colonies were sequenced and similarity searches against the publicly available databases revealed a marigold gene with similarity to the *E. coli* MinD protein. This *Tagetes* DNA sequence, designated *TeMinD*, is shown in SEQ ID NO:3 and its deduced amino acid sequence,

designated TeMinD, is shown in SEQ ID NO:4.

Construction of Chimeric Sense and Antisense MinD Protein Coding Sequences.

The MZF18 clone (AB009056) was obtained from the *Arabidopsis* Biological Research Center in Columbus, Ohio. The region corresponding to the *AtminD1* ORF was amplified from MZF18 with Deep Vent Polymerase (New England BioLab) using the following primers: forward primer, 5'-CCGAATTCGAAGCAGCAGCACTATCAATGG-3'; reverse primer 5'-CGGAATTCGATCCGTTTGCCATTTAGCC-3'. Both primers incorporated recognition sites for *Eco*RI. The PCR product was sequenced in its entirety to ensure that no mutations had been introduced, and ligated in both orientations into pBluescript (Stratagene). The plasmid with the 5' end of the insert nearest the T3 promoter was designated KG405; the plasmid with 5' end of the insert nearest the T7 promoter was designated KG406. The plasmids were maintained in a *minCDE* deletion strain of *E. coli,* RC3F. For the transgenic constructs, the *Eco*RI-restricted PCR fragment was ligated into the *Eco*RI cloning site of pART7 behind the CaMV 35S promoter in either the sense or antisense orientation. The transgenes were then excised from the resulting plasmids with *NotI* and ligated into the *NotI* cloning site in the binary transformation vector pART27, yielding plasmids KG402 containing the *AtMinD1* antisense construct, and KG404 containing the *AtMinD1* sense construct. Both transformation vectors also included a selectable marker from pART27 conferring plant resistance to kanamycin.

Characterization of MinD Synthesis in Plants.

Relative to the MinD proteins from *C. vulgaris* and several prokaryotes, AtMinD contains an amino terminal extension with features common to chloroplast transit peptides. These include alanine as the second residue, a relatively high proportion of hydroxylated amino acids, and few acidic residues. An in vitro chloroplast import assay was performed to determine whether this extension was able to function as a chloroplast targeting sequence.

Plasmid KG405, described above, was linearized with BamHI and transcribed

using T3 RNA polymerase. A plasmid containing the prSS control encoding the small subunit of pea RuBP carboxylase was linearized with *PstI* and transcribed with SP6 RNA polymerase. The resulting transcripts were translated in a rabbit reticulocyte lysate translation system (Promega) containing [^{35}S]methionine (DuPont/NEN). Import reactions were carried out using chloroplasts isolated from 8- to 12-day-old pea seedlings (*Pisum sativum* var. *Little Marvel*, Olds Seed Company, Madison, WI) and purified over a Percoll gradient. Intact chloroplasts were reisolated and resuspended in import buffer (330 mM sorbitol, 50 mM Hepes/KOH, pH 8.0) at a concentration of 1 mg chlorophyll/ml. Thermolysin treatment of import products was performed and import products were analyzed by SDS-PAGE and fluorography.

The in vitro transcription of the *AtMinD1* ORF, followed by the in vitro translation of the resulting transcript in the presence of [^{35}S]methionine, yielded a full-length, radiolabeled translation product that migrated at 39.7 kD, somewhat above its calculated mass. When added to the isolated pea chloroplasts, the translation product was processed to a smaller form migrating at 35.6 kD. The processed form of the protein was soluble following import, and was fully protected from a post-import treatment with the protease thermolysin. In a control set of reactions, the small subunit of pea RuBP carboxylase/oxygenase, a soluble stromal protein, behaved identically. These results provide strong evidence that AtMinD, like the *Arabidopsis* FtsZ1, is synthesized as a precursor on cytosolic ribosomes and posttranslationally targeted to the chloroplast where it is processed to a mature form.

Effect of Antisense Repression of MinD on Chloroplast Size and Number.

To demonstrate that the *AtMinD1* gene functions in the placement of the plastid-localized FtsZ ring and the positioning of the plastid division machinery, the transformation vector KG402, containing the *AtMinD1* antisense construct, was introduced into *Arabidopsis* plants (ecotype Columbia (Col-0)) by *Agrobacterium*-mediated transformation.

The *Arabidopsis* plants utilized arose from seeds sown on a Supersoil potting mix and vermiculite in a ratio of 3:1. The seeds were incubated at 4°C in the dark for two days before being moved to growth chambers and grown at 22°C with 16 hrs of

daylight. The age of the plants was calculated from the first day of their transfer to growth chambers.

Agrobacterium-mediated transformation was performed by a freeze-thaw method using *Agrobacterium tumefaciens* C58 (GV3101). The plasmids were checked for rearrangements following transfer to *Agrobacterium* by back-transformation to *E. coli* and restriction analysis. The transformation vectors were introduced into the *Arabidopsis* plants using the floral dip procedure. Transformants were selected by germination in nutrient medium containing 50 or 100 mg/l kanamycin and transplanted to soil 7-10 d after germination for propagation and analysis. Kanamycin resistant (kan^r) plants that originated from different pots were assumed to be derived from independent T-DNA insertion events for the purposes of phenotype characterization.

T_1 seeds were harvested from the inoculated plants, and transformants were selected on the basis of their resistance to the antibiotic. Leaf tissue from kan^r plants were examined microscopically for effects on chloroplast size and number. Plants from 19 different pots were analyzed, ensuring that the phenotypes observed were the result of a minimum of 19 independent transformation events. Based on recent studies of T-DNA insertion patterns in *Arabidopsis* transformed by a similar procedure, it is likely that most of the kan^r T_1 individuals, including those originating from the same pot, represented independent insertion events.

The phenotypes of the antisense transformants were initially investigated by examination of mesophyll cells from first leaves of 23-day-old T_1 plants. In wild-type plants, the leaves at this stage of development are fully expanded such that the cells have accumulated their full complement of approximately 100 chloroplasts, all of which fall within a narrow range of sizes (Figure 3). Among the 164 kan^r individuals examined from the 19 antisense transformations, 90 (55%) exhibited phenotypes that differed noticeably from wild type. Among these, 66 (73%) displayed a striking degree of heterogeneity in the sizes of the chloroplasts within a single mesophyll cell. This heterogeneity was evident both from visual inspection of the mesophyll cells under the microscope and from measurements of the frequency with which chloroplasts of different sizes were observed in the same cell (Figure 3, panels B-D). The plastid size heterogeneity was even more pronounced in smaller cells from younger leaves in which

chloroplasts are not yet fully expanded as they are in 23-day-old leaves.

Chloroplast numbers per unit cell area were also quite variable in the antisense plants, in contrast with wild type in which the number of chloroplasts per cell is tightly correlated with cell size. However, the chloroplasts were consistently fewer in number and larger in size than in wild type cells, suggesting a reduced number of plastid division events in most of the *AtMinD1* antisense lines. The phenotypes observed in the T_1 generation were also observed in T_2 and T_3 progeny.

Although chloroplast numbers in cells from antisense plants were consistently lower and the chloroplast sizes far less uniform than in wild type, the linear relationship between the total chloroplast plan area and the total mesophyll cell plan area in the antisense lines was approximately the same as in wild type. This finding indicates that the reduced chloroplast numbers were compensated for by corresponding increases in chloroplast expansion so that total chloroplast volume was conserved. Similar results have been shown for other perturbations in chloroplast number and/or expansion .

A relatively small proportion (18%) of T_1 plants with visually detectable phenotypes under microscope displayed less heterogeneity in chloroplast size within single cells. Instead, the mesophyll tissue in these plants comprised a mixture of cells containing either wild-type numbers and sizes of chloroplasts or only a few large chloroplasts. Because the affected cells contained fewer chloroplasts than the number of proplastids present in leaf primordia, these observations suggest a significant inhibition of both proplastid and chloroplast division in some cells, but not in others.

Under the growth conditions used for the experiments, plants expressing the *AtMinD1* antisense transgene grew more rapidly than wild type in the early stages of development (first leaves appeared earlier), but inflorescences appeared a few days later. This difference was evident through the T_2 and T_3 generations. In other aspects of growth and development, the antisense plants did not differ noticeably from wild type, however, careful measurements of growth parameters may reveal other subtle differences.

To confirm that the transgenic phenotypes resulted from reduced expression of the endogenous *AtminD1* gene, a northern blot of poly(A)$^+$ RNA isolated from antisense and wild type plants was probed with a radiolabeled RNA probe specific for *AtMinD1*.

Total RNA was isolated from 23-27-day-old plants as described previously using 1 g of leaf tissue from independent transgenic lines (T_3) or from wild type to determine the expression levels of the AtMinD protein coding sequence. Only transgenic individuals exhibiting plastid size heterogeneity for the *AtMinD1* antisense plants were used for RNA isolation. Poly(A)$^+$ RNA was isolated with Oligotex resin (Qiagen) according to the manufacturer using total RNA as starting material, and quantified by measuring absorbance at 260 and 280 nm. Poly(A)$^+$ RNA gel blots were prepared as described previously using nylon membrane (Micron Separations, Inc.). An RNA probe for hybridization specifically to sense *AtMinD1* mRNA was prepared by linearizing KG406 with HindIII, and carrying out an in vitro transcription reaction in the presence of ^{32}P-UTP (800 Ci/mmol; ICN, Costa Mesa, CA) as described previously, but using T7 RNA polymerase (New England BioLab). Blots were hybridized overnight and washed in 0.2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 68°C.

The probe hybridized to two transcripts of about 1.1 and 1.7 kb, the smaller of which was more abundant. The probe remained bound to both transcripts when the blot was washed at very high stringency, indicating the two mRNAs were derived from either the same gene or from two closely related genes. Based on the size of the *AtMinD1* open reading frame (978 bp), it was expected that *AtMinD1* is represented by at least the smaller transcript. The levels of both transcripts were significantly reduced in the antisense plants when compared with wild type, indicating that the heterogeneity in chloroplast size and number in these plants was the result of reduced *AtMinD1* expression.

To determine whether the heterogeneity in chloroplast size observed in the antisense repression of *AtMinD1* could be the result of asymmetric chloroplast division, petal tissue from flowers of the transgenic plants were examined. Normally, in leaves of dicotyledonous plants, the division of chloroplasts is rapid and is not synchronized. Consequently, it can be difficult to observe chloroplasts in the process of division, particularly in the *AtMinD1* antisense plants in which chloroplast numbers are reduced. However, in the present case, a high frequency of constricted plastids were documented in the *Arabidopsis* petals and easily viewed because the plastids were less densely packed than in mesophyll cells.

In many of the constricted plastids, the constriction was noticeably displaced from the center. This is in marked contrast from wild type plants in which petal plastids almost always appear to be constricted in the center. Asymmetric constriction of chloroplasts in leaf epidermal cells in the transgenic lines was also observed. Collectively, this data suggests that the chloroplast size variability in the *AtMinD1* antisense plants results at least partially from asymmetric plastid division.

Effect of MinD Overexpression in Transgenic Plants.

To further analyze the role of *AtMinD1* in plastid division, the transformation vector KG404, containing the sense *AtminD1* construct under control of the CaMV 35S promoter, was introduced into *Arabidopsis* plants by *Agrobacterium*-mediated transformation as described above.

The phenotypes of 82 kan^r T_1 individuals representing at least 13 independent transformation events were investigated microscopically. The predominant phenotype, observed in 52 (73%) of the 71 T_1 plants having phenotypes that were clearly distinguishable from wild type, was a dramatically reduced number of greatly enlarged chloroplasts in comparison to wild type. Cells in most of these plants appeared to contain five or fewer chloroplasts, and many had only a single large chloroplast. This phenotype contrasted with that observed in most of the antisense plants, in which the chloroplasts were generally more numerous, and indicates a more severe inhibition of plastid division. The phenotype was also inherited in the T_2 and T_3 progeny. Because the number of chloroplasts in mesophyll cells from the *AtMinD1* overexpression lines was less than the number of proplastids present in the cells of the shoot apical meristem, it is believed that a disruption of both proplastid and chloroplast division in these plants occurred.

To confirm that the transgenic phenotypes resulted from overexpression of the endogenous *AtminD1* gene, a northern blot of poly(A)$^+$ RNA isolated from sense and wild type plants was performed using a radiolabeled RNA probe specific for *AtMinD1*. Total RNA was isolated from 23- to 27-day-old plants as described previously using 1 g of leaf tissue from independent transgenic lines (T_3) or from wild type to determine the expression levels of the AtMinD protein coding sequence. Only transgenic individuals

exhibiting severely reduced numbers of chloroplasts for the *AtMinD1* sense plants were used for RNA isolation. Northern blot analysis confirmed that the severe disruption in chloroplast division was accompanied by *AtminD1* overexpression.

The remaining 19 (29%) T_1 individuals among the 71 that differed obviously from wild type had less severe defects in plastid division. Most of these resembled the antisense plants, having variable numbers and sizes of chloroplasts. It has not been determined whether this phenotype is indeed the result of *AtMinD1* overexpression, although it parallels findings in *E. coli* that suggest overexpression of *minD* induces minicell formation. However, this phenotype is also consistent with cosuppression of endogenous *AtMinD1* gene expression. The *AtMinD1* sense lines grew somewhat more slowly and did not grow as large as the wild type or antisense plants. They also began flowering about 3 days earlier on average.

Taken together, these results indicate that either decreased or increased numbers of chloroplasts can be obtained in transgenic plants by manipulation of MinD expression levels. Manipulation of the size and shape of chloroplasts may also be obtained.

CLAIMS

I CLAIM:

1. A transgenic plant comprising in its genome an artifical genetic construct comprising a sense or antisense MinD protein coding sequence and a promoter which promotes expression of the MinD protein coding sequence in cells of the plant, wherein expression of the sequence in the plant cause alteration in the size, shape and/or number of plastids in plant cells of the plant as compared to non-transgenic plants of the species.

2. The plant of Claim 1, wherein the coding sequence is selected from the group consisting of an Arabidopsis MinD protein coding sequence and a Tagetes MinD protein coding sequence.

3. The plant of Claim 1, wherein the coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

4. The plant of Claim 1, wherein the construct comprises in 5' to 3' order a CaMV 35S promoter, a MinD protein coding sequence, and an OCS terminator.

5. The plant of Claim 4, wherein the coding sequence is selected from the group consisting of an Arabidopsis MinD protein coding sequence and a Tagetes MinD protein coding sequence.

6. The plant of Claim 4, wherein the coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

7. The plant of Claim 1, wherein the plastids are chloroplasts.

8. A DNA sequence comprising the sequence of SEQ ID NO:1.

9. A DNA sequence comprising the sequence of SEQ ID NO:3.

10. Seed of the plant of Claim 1.

11. A plant comprising in its genome a transgene comprising a sense or antisense MinD gene which causes the plant to have an altered number of plastids as compared to plants of the same species with the transgene.

12. Seeds of the plant of Claim 11.

13. A plant as claimed in Claim 11 wherein the coding sequence of the MinD gene is selected from the group consisting of AtMinD and TeMinD.

14. A plant seed comprising in its genome a genetic construct comprising a sense or antisense MinD protein coding sequence and a promoter, not natively associated with the MinD protein coding sequence, which promotes expression of the MinD protein coding sequence in the plant, wherein expression of the sequence in the plant cause alteration in the size, shape and/or number of plastids in plant cells of the plant as compared to nontransgenic plants of the species.

15. The plant of Claim 14, wherein the coding sequence is selected from the group consisting of an Arabidopsis MinD protein coding sequence and a Tagetes MinD protein coding sequence.

16. The plant of Claim 14, wherein the coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

17. The plant of Claim 14, wherein the construct comprises in 5' to 3' order a CaMV 35S promoter, a MinD protein coding sequence, and an OCS terminator.

18. The plant of Claim 17, wherein the coding sequence is selected from the group consisting of an Arabidopsis MinD protein coding sequence and a Tagetes MinD protein coding sequence.

19. The plant of Claim 17, wherein the coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

20. A genetic construct comprising a MinD protein coding sequence in either a sense or antisense orientation and a promoter that promotes expression of the sequence in plants, the promoter not being natively associated with the protein coding sequence.

21. The construct of Claim 20, wherein the MinD protein coding sequence is selected from the group consisting of an Arabidopsis MinD protein coding sequence and a Tagetes MinD protein coding sequence.

22. The construct of Claim 20, wherein the coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

23. The construct of Claim 20, wherein the promoter is a CaMV 35S promoter.

24. A method for altering the size, shape and/or number of plastids in plant cells comprising the steps of constructing a genetic construct comprising a MinD protein coding sequence in either sense or antisense orientation and a promoter, not natively associated with the MinD protein coding sequence, which promotes expression of the MinD protein coding sequence in plants, introducing the genetic construct into a plant, selecting a plant that has received a copy of the genetic construct, and growing the plant under conditions that allow expression of the gene.

25. The method of Claim 24, wherein the coding sequence is selected from the group consisting of an Arabidopsis MinD protein coding sequence and a Tagetes MinD protein coding sequence.

26. The method of Claim 24, wherein the coding sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

27. A DNA sequence isolated from its native genome comprising a plant MinD gene.

28. The DNA sequence of Claim 27, wherein the DNA sequence is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.

ABSTRACT

Disclosed are MinD protein coding sequences that play a critical role in regulating the division of plastids in plants. Also disclosed is a method for obtaining transgenic plants with novel phenotypes, characterized by alterations in plastid shape, number and/or size.

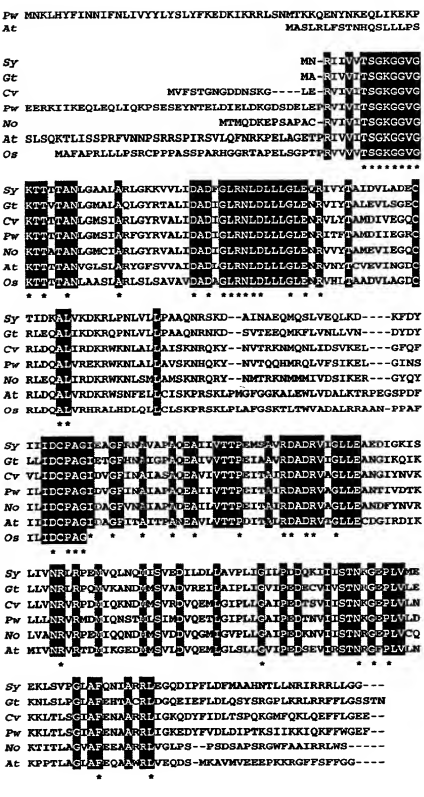
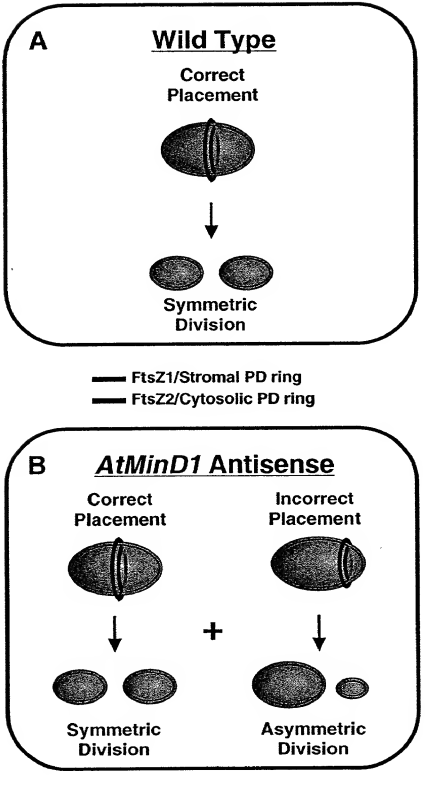


FIG 1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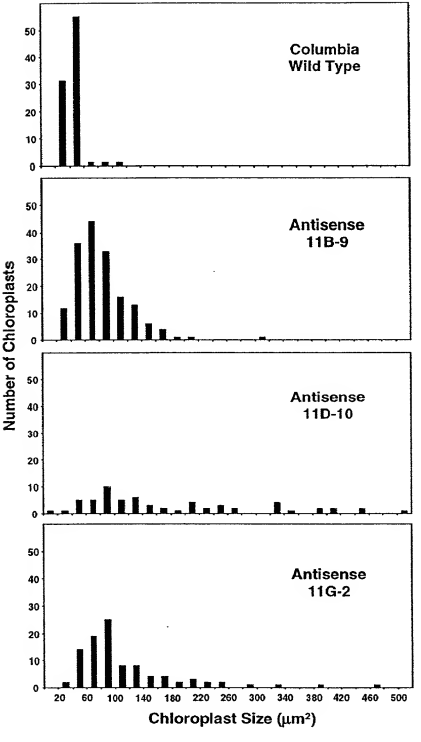
Correct Placement
Symmetric Division
FtsZ1/Stromal PD ring
FtsZ2/Cytosolic PD ring
B
AtMinD1 Antisense
Correct Placement
Incorrect Placement
+
Symmetric Division
Asymmetric Division

FIG 2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Chloroplast Size (μm²)
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30
40
50
20
60
100
140
180
220
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300
340
380
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FIG 3

EXPRESS MAIL LABEL NO. EK290771354US

PTO/SB/01 (6-95)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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0010/PTO Rev. 6/95 — U.S. Department of Commerce Patent and Trademark Office

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing OR [ ] Declaration Submitted after Initial Filing

Attorney Docket Number	920905.90041
First Named Inventor	Katherine W. Osteryoung
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MANIPULATION OF MIN GENES IN PLANTS

(Title of the Invention)

the specification of which

[X] is attached hereto

OR

[ ] was filed on (MM/DD/YYYY) _____ as United States Application Number or PCT International Application Number _____ and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign applications numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/130,403	04/19/99

[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

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DECLARATION

I hereby claim benefit under Title 35, United States Code §120 of any United States application(s), or §365(C) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT international application in the manner provided in the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

[ ] Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and all continuation and divisional applications based thereon, and to transact all business in the Patent and Trademark Office connected therewith:

[ ] Firm Name _____ Customer Number or label _____

OR

[X] List attorney(s) and/or agent(s) name and registration number below

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Carl R. Schwartz	29,437	Jill A. Fahrlander	42,518
Gregory A. Nelson	30,577	Scott D. Paul	42,984
Keith M. Baxter	31,233	Daniel G. Radler	43,028
John D. Franzini	31,356	Steven J. Wietrzny	44,402

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of Sole or First Inventor: [ ] A petition has been filed for this unsigned inventor

Given	Katherine	Middle	W.	Family	Osteryoung	Suffix	

Inventor's Signature _____ Date _____

Residence: _____ State _____ Country _____ Citizenship _____

Post Office _____

Post Office _____

City _____ State _____ Zip _____ Country _____ Applicant Authority _____

[ ] Additional inventors are being named on supplemental sheet(s) attached hereto

SEQUENCE LISTING

<110> Osteryoung, Katherine W.

<120> Manipulation of Min Genes in Plants

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Pro Ser Ser Leu Ser Gln Lys Thr Leu Ile Ser Ser Pro Arg Phe Val
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    130                 135                 140

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gga ttg tca ttg ctt ggt gta att cct gaa gat tct gag gtt att cga   816
Gly Leu Ser Leu Leu Gly Val Ile Pro Glu Asp Ser Glu Val Ile Arg
            260                 265                 270

agc acg aat cga ggg ttt ccg ctt gtt ctg aat aag cct cct acg ctt   864
Ser Thr Asn Arg Gly Phe Pro Leu Val Leu Asn Lys Pro Pro Thr Leu
        275                 280                 285

gcg gga ttg gcg ttt gag cag gcg gct tgg aga ctc gtt gag caa gat   912
Ala Gly Leu Ala Phe Glu Gln Ala Ala Trp Arg Leu Val Glu Gln Asp
    290                 295                 300

agt atg aag gct gtt atg gtg gag gaa gaa cct aag aaa cgt ggc ttc   960
Ser Met Lys Ala Val Met Val Glu Glu Glu Pro Lys Lys Arg Gly Phe
305                 310                 315                 320

ttc tct ttc ttt ggc ggc                                          978
Phe Ser Phe Phe Gly Gly
                325
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<210> 2
<211> 326
<212> PRT
<213> Arabidopsis thaliana

<400> 2

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Met Ala Ser Leu Arg Leu Phe Ser Thr Asn His Gln Ser Leu Leu Leu
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Pro Ser Ser Leu Ser Gln Lys Thr Leu Ile Ser Ser Pro Arg Phe Val
            20                  25                  30

Asn Asn Pro Ser Arg Arg Ser Pro Ile Arg Ser Val Leu Gln Phe Asn
        35                  40                  45

Arg Lys Pro Glu Leu Ala Gly Glu Thr Pro Arg Ile Val Val Ile Thr
    50                  55                  60

Ser Gly Lys Gly Gly Val Gly Lys Thr Thr Thr Thr Ala Asn Val Gly
65                  70                  75                  80

Leu Ser Leu Ala Arg Tyr Gly Phe Ser Val Val Ala Ile Asp Ala Asp
                85                  90                  95

Leu Gly Leu Arg Asn Leu Asp Leu Leu Leu Gly Leu Glu Asn Arg Val
            100                 105                 110

Asn Tyr Thr Cys Val Glu Val Ile Asn Gly Asp Cys Arg Leu Asp Gln
        115                 120                 125

Ala Leu Val Arg Asp Lys Arg Trp Ser Asn Phe Glu Leu Leu Cys Ile
    130                 135                 140

Ser Lys Pro Arg Ser Lys Leu Pro Met Gly Phe Gly Gly Lys Ala Leu
145                 150                 155                 160

Glu Trp Leu Val Asp Ala Leu Lys Thr Arg Pro Glu Gly Ser Pro Asp
                165                 170                 175

Phe Ile Ile Ile Asp Cys Pro Ala Gly Ile Asp Ala Gly Phe Ile Thr
            180                 185                 190

Ala Ile Thr Pro Ala Asn Glu Ala Val Leu Val Thr Thr Pro Asp Ile
        195                 200                 205

Thr Ala Leu Arg Asp Ala Asp Arg Val Thr Gly Leu Leu Glu Cys Asp
    210                 215                 220

Gly Ile Arg Asp Ile Lys Met Ile Val Asn Arg Val Arg Thr Asp Met
225                 230                 235                 240

Ile Lys Gly Glu Asp Met Met Ser Val Leu Asp Val Gln Glu Met Leu
                245                 250                 255

Gly Leu Ser Leu Leu Gly Val Ile Pro Glu Asp Ser Glu Val Ile Arg
            260                 265                 270
```

```
Ser Thr Asn Arg Gly Phe Pro Leu Val Leu Asn Lys Pro Pro Thr Leu
        275                 280                 285

Ala Gly Leu Ala Phe Glu Gln Ala Ala Trp Arg Leu Val Glu Gln Asp
    290                 295                 300

Ser Met Lys Ala Val Met Val Glu Glu Glu Pro Lys Lys Arg Gly Phe
305                 310                 315                 320

Phe Ser Phe Phe Gly Gly
                325
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<210> 3
<211> 1182
<212> DNA
<213> Tagetes erecta

<220>
<221> CDS
<222> (50)..(934)

<400> 3

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                                                      Met Thr Ser
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ctg agg ttt cta aca gaa ccc tca ctt gta tgc tca tcc act ttc ccc   106
Leu Arg Phe Leu Thr Glu Pro Ser Leu Val Cys Ser Ser Thr Phe Pro
      5                  10                  15

aca ttc aat ccc cta cac aaa acc cta act aaa cca aca cca aaa ccc   154
Thr Phe Asn Pro Leu His Lys Thr Leu Thr Lys Pro Thr Pro Lys Pro
 20                  25                  30                  35

tac cca aag cca cca cca att cgc tcc gtc ctt caa tac aat cgc aaa   202
Tyr Pro Lys Pro Pro Pro Ile Arg Ser Val Leu Gln Tyr Asn Arg Lys
                 40                  45                  50

cca gag ctc gcc gga gac act cca cga gtc gtc gca atc gac gcc gac   250
Pro Glu Leu Ala Gly Asp Thr Pro Arg Val Val Ala Ile Asp Ala Asp
             55                  60                  65

gtt ggt cta cgt aac ctc gat ctt ctt ctc ggt ctc gaa aac cgc gtc   298
Val Gly Leu Arg Asn Leu Asp Leu Leu Leu Gly Leu Glu Asn Arg Val
         70                  75                  80

aat tac acc gtc gtt gaa gtt ctc aac ggc gat tgc aga ctc gac caa   346
Asn Tyr Thr Val Val Glu Val Leu Asn Gly Asp Cys Arg Leu Asp Gln
     85                  90                  95

gcc cta gtt cgt gat aaa cgc tgg tca aat ttc gaa ttg ctt tgt att   394
Ala Leu Val Arg Asp Lys Arg Trp Ser Asn Phe Glu Leu Leu Cys Ile
100                 105                 110                 115

tca aaa cct agg tca aaa ttg cct tta gga ttt ggg gga aaa gct tta   442
Ser Lys Pro Arg Ser Lys Leu Pro Leu Gly Phe Gly Gly Lys Ala Leu
                120                 125                 130
```

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gtt tgg ctt gat gca tta aaa gat agg caa gaa ggt tgc ccg gat ttt   490
Val Trp Leu Asp Ala Leu Lys Asp Arg Gln Glu Gly Cys Pro Asp Phe
                135                 140                 145

ata ctt ata gat tgt cct gca ggt att gat gcc ggg ttc ata acc gcc   538
Ile Leu Ile Asp Cys Pro Ala Gly Ile Asp Ala Gly Phe Ile Thr Ala
        150                 155                 160

att aca ccg gct aac gaa gcc gta tta gtt aca aca cct gat att act   586
Ile Thr Pro Ala Asn Glu Ala Val Leu Val Thr Thr Pro Asp Ile Thr
    165                 170                 175

gca ttg aga gat gca gat aga gtt aca ggc ttg ctt gaa tgt gat gga   634
Ala Leu Arg Asp Ala Asp Arg Val Thr Gly Leu Leu Glu Cys Asp Gly
180                 185                 190                 195

att agg gat att aaa atg att gtg aac aga gtt aga act gat ttg ata   682
Ile Arg Asp Ile Lys Met Ile Val Asn Arg Val Arg Thr Asp Leu Ile
                200                 205                 210

agg ggt gaa gat atg atg tca gtt ctt gat gtt caa gag atg ttg gga   730
Arg Gly Glu Asp Met Met Ser Val Leu Asp Val Gln Glu Met Leu Gly
            215                 220                 225

ttg tca ttg ttg agt gat acc cga gga ttc gaa gtg att cgg agt acg   778
Leu Ser Leu Leu Ser Asp Thr Arg Gly Phe Glu Val Ile Arg Ser Thr
        230                 235                 240

aat aga ggg ttt ccg ctt gtg ttg aac aag cct ccg act tta gca gga   826
Asn Arg Gly Phe Pro Leu Val Leu Asn Lys Pro Pro Thr Leu Ala Gly
    245                 250                 255

ttg gca ttt gag cag gct gct tgg aga ttg gtt gag caa gat agc atg   874
Leu Ala Phe Glu Gln Ala Ala Trp Arg Leu Val Glu Gln Asp Ser Met
260                 265                 270                 275

aag gct gtg atg gtg gag gaa gaa cct aaa aag agg gga ttt ttc tcg   922
Lys Ala Val Met Val Glu Glu Glu Pro Lys Lys Arg Gly Phe Phe Ser
                280                 285                 290

ttt ttt gga ggt tagtgatcga attcgtttgaa tcgttgagtt gggtttgttt       974
Phe Phe Gly Gly
            295

tggtggagaa atgtgtcttg tttgttcatg taggagctgc tatgtgtcac ttgaaatgtt 1034

atgtgtacag taagctgata aggattgttt taattcagtt ttcagagaga aaattagaat 1094

tgtagcaact tttcatttga tcaattcaat tgtatttctt tggttcagtg atgaattttt 1154

actcaaaatc aaaaaaaaaa aaaaaaaa                                    1182
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<210> 4
<211> 295
<212> PRT
<213> Tagetes erecta

<400> 4

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Met Thr Ser Leu Arg Phe Leu Thr Glu Pro Ser Leu Val Cys Ser Ser
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Thr Phe Pro Thr Phe Asn Pro Leu His Lys Thr Leu Thr Lys Pro Thr
            20                  25                  30

Pro Lys Pro Tyr Pro Lys Pro Pro Pro Ile Arg Ser Val Leu Gln Tyr
        35                  40                  45

Asn Arg Lys Pro Glu Leu Ala Gly Asp Thr Pro Arg Val Val Ala Ile
    50                  55                  60

Asp Ala Asp Val Gly Leu Arg Asn Leu Asp Leu Leu Leu Gly Leu Glu
65                  70                  75                  80

Asn Arg Val Asn Tyr Thr Val Val Glu Val Leu Asn Gly Asp Cys Arg
                85                  90                  95

Leu Asp Gln Ala Leu Val Arg Asp Lys Arg Trp Ser Asn Phe Glu Leu
            100                 105                 110

Leu Cys Ile Ser Lys Pro Arg Ser Lys Leu Pro Leu Gly Phe Gly Gly
        115                 120                 125

Lys Ala Leu Val Trp Leu Asp Ala Leu Lys Asp Arg Gln Glu Gly Cys
    130                 135                 140

Pro Asp Phe Ile Leu Ile Asp Cys Pro Ala Gly Ile Asp Ala Gly Phe
145                 150                 155                 160

Ile Thr Ala Ile Thr Pro Ala Asn Glu Ala Val Leu Val Thr Thr Pro
                165                 170                 175

Asp Ile Thr Ala Leu Arg Asp Ala Asp Arg Val Thr Gly Leu Leu Glu
            180                 185                 190

Cys Asp Gly Ile Arg Asp Ile Lys Met Ile Val Asn Arg Val Arg Thr
        195                 200                 205

Asp Leu Ile Arg Gly Glu Asp Met Met Ser Val Leu Asp Val Gln Glu
    210                 215                 220

Met Leu Gly Leu Ser Leu Leu Ser Asp Thr Arg Gly Phe Glu Val Ile
225                 230                 235                 240

Arg Ser Thr Asn Arg Gly Phe Pro Leu Val Leu Asn Lys Pro Pro Thr
                245                 250                 255

Leu Ala Gly Leu Ala Phe Glu Gln Ala Ala Trp Arg Leu Val Glu Gln
            260                 265                 270
```

```
Asp Ser Met Lys Ala Val Met Val Glu Glu Glu Pro Lys Lys Arg Gly
        275                 280                 285

Phe Phe Ser Phe Phe Gly Gly
    290                 295
```